Osteoarthritis and Cartilage 23 (2015) 2279–2287

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Autophagy protects chondrocytes from glucocorticoids-induced apoptosis via ROS/Akt/FOXO3 signaling

C. Shen, G.-Q. Cai, J.-P. Peng, X.-D. Chen*

Department of Orthopedic, Xin-hua Hospital, China

ARTICLE INFO

Article history:
Received 7 January 2015
Accepted 29 June 2015

Keywords:
Autophagy
Chondrocytes
Forkhead-box class O3
Dexamethasone

SUMMARY

Objective: Glucocorticoids (GCs) have been widely used in the management of osteoarthritis (OA) and rheumatoid arthritis (RA). Nevertheless, there has been some concern about their ability of increasing reactive oxygen species (ROS) in the cartilage. Forkhead-box class O (FOXO) transcription factors have been proved to have a protective role in chondrocytes through regulation of autophagy and defending oxidative stress. The objective of this study was to investigate the role of FOXO3 in Dex-induce up-regulation of ROS.

Design: Healthy cartilages debris from six patients were used for chondrocytes culture. After the treatment of dexamethasone (Dex), the ROS levels, autophagic flux, the expression of FOXO3 in chondrocytes were measured. RNA interference technique was also used to determine the role of FOXO3 in Dex-induced autophagy. The metabolism of the extra-cellular matrix was also investigated.

The results: Dex increased intracellular ROS level, the expression of Akt, FOXO3 as well as autophagy flux in human chondrocytes. The expression of aggrecanases also increased after the treatment of Dex. Catalase, the ROS scavenger, suppressed Dex-induced up-regulation of autophagy flux and expression of aggrecanases and Akt. MK-2206 and LY294002, the PI3K/Akt inhibitors, repressed Dex-induced up-regulation of FOXO3. Silencing FOXO3 resulted in down-regulation of Dex-induced autophagy. Moreover, knockdown of FOXO3 increased Dex-induced apoptosis as well as ROS levels in chondrocytes. In addition, up-regulation of autophagy by Rapamycin resulted in decreasing ROS level in chondrocytes.

Conclusion: Dex could advance the degenerative process in cartilage. Autophagy was induced in response to Dex-induced up-regulation of ROS via ROS/Akt/FOXO3 signal pathway.

Introduction

Local administration of glucocorticoids (GCs) has been used in the management of osteoarthritis (OA), rheumatoid arthritis (RA) as well sports injuries[1,2]. GC works via its receptor and inhibits local immunity reaction[3]. Therefore, GC plays a critical role in relieving the symptoms in the patients. However, there has been a growing body of evidences showed that GCs had a degenerative-effect on several collagen-producing tissues, such as bone, tendons and skin[4–7]. Accordingly, interests have focused on how GCs led to degeneration in these tissues. However, the mechanism of GC-induced degeneration is largely unknown. Recently, it has been reported that the GC could dysregulate the mitochondria function and therefore induce apoptosis[8].

Dysregulation of mitochondria could result in an increase of intracellular reactive oxygen species (ROS)[9]. ROS plays a crucial role in the homeostasis of cell energetic cycling and extra-cellular matrix metabolism. In non-immune cells, endogenous ROS generated from mitochondria in response to pro-inflammatory cytokines and mechanical stress[10]. Nevertheless, excessive ROS tightly associated with degenerative and aging diseases, such as OA, Alzheimer's disease and Huntington's disease[11,12]. Recent studies demonstrated that oxidative stress caused by ROS could induce apoptosis in chondrocytes[8,13,14]. Besides, ROS also contributes to the degradation of cartilage by up-regulation of the expression of matrix metalloproteinases (MMPs)[12,15]. Therefore, increasing level of ROS would advance the pathological process of OA.

Transcription factors of the Forkhead box O class (FOXOs) play an important role in maintaining the intracellular ROS

* Address correspondence and reprint requests to: X.-D., Chen, Department of Orthopedic Surgery, Xinhua Hospital, 1665 Kongjiang Road, 200092, Shanghai, China. Tel: 86-21-25078999-7950; Fax: 86-21-25078999-7970.

E-mail addresses: Samsonov@126.com (C. Shen), Yiranh@yeah.net (G.-Q. Cai), hadesshen1109@yahoo.com (J.-P. Peng), duchemin@163.com (X.-D. Chen).

http://dx.doi.org/10.1016/j.joca.2015.06.020
1063-4584/

balance[4,16–18]. The increasing level of ROS could enhance the expressions of FOXOs[17]. FOXOs, in turn, up-regulated the expressions of anti-oxidant genes, such as SOD2 and sestrin family[19]. In contrast, dysregulation of FOXOs would result in an increase in both cell death rate and intracellular level of ROS in chondrocytes[17]. Recently, FOXOs have been reported to be involved in regulation of autophagy during the pathological processes of aging and OA[16].

Autophagy is a critical energy recycling mechanism of eukaryotic cells[20]. We and other authors had demonstrated that autophagy could protect the chondrocytes or fibro-chondrocytes against the ROS and inflammatory cytokines[21–24]. Recently, there was study showed that autophagy protected chondrocytes from GC-induced apoptosis[25]. However, the mechanism of GC-induced autophagy was largely unknown. In the current study, we investigated the potential signal pathway of GC-induced autophagy. Our results indicated that dexamethasone (Dex), a synthetic GC, induced autophagy via ROS/Akt/FOXO3 pathway.

Material and methods

Cells isolation and preparation

After approval by the institutional review board of Xin-Hua hospital (School of medicine, Shanghai Jiao Tong University), healthy cartilages debris were obtained from six female patients (age 20.5 ± 4.7) during the femoroacetabular impingement surgeries. To address the potential changes of the phenotypes of the chondrocytes from these patients, the mRNA expressions of collagen type I and II in these chondrocytes were investigated. Health cartilages debris from three female patients (age 15, 24, 27 years) during osteochondral autograft transplantation surgery were used as control. Before the surgery, every patient was well informed and an instant consent was assigned. The debris cartilages were used for cell culture. The tissue was then minced into 1 mm^3 and digested in 0.1% collagenase type II (Sigma–Aldrich, St Louis, MO, USA) for 4–5 h at 37°C. Then the debris was seeded in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, USA) medium with 10% fetal bovine serum (FBS, Gibco, USA) in a 37°C, 5% CO_2 environment. After reaching 70% confluence, the primary passage cells were harvested and replanted. When the first-passage cells reaching 90% confluence, in order to synchronize the cells, the cultured medium was changed to DMEM/F-12 with 1% FBS and antibiotics for 12 h before further experiments. The real-time PCR results showed that there was no significant difference in the mRNA expression of collagen type I and II between the experimental group and control group (Table I).

ROS measurement

The intracellular levels of chondrocytes were measured using a ROS detection kit (Beyotime, Haimen, China). Cells in 6-well culture plates were incubated with different concentrations of Dex for 24 h. Harvested cells were washed with serum-free culture medium and incubated with 10 μM DCFH-DA at 37°C for 20 min. Then the distribution of DCF fluorescence was recorded by a fluorospectrophotometer at an excitation wavelength of 488 NM and at an emission wavelength of 535 nm. The ROS level of the chondrocytes cultured in 10%FBS was used as control.

Western blotting analysis

The protein of treated chondrocytes was separated on SDS-PAGE and then transferred to a polyvinylidene difluoride membrane as our previous described[22]. The membranes were blocked with 5% BSA followed by immunoblotting with LC3 (1:3000, Abcam, UK), Beclin-1 (1:300, Abcam, UK), *p*-Akt (1:500, Abcam, UK), *p*-P70S6K (1:500, Abcam, UK), FOXO3 (1:500, Abcam, UK), Bcl-2 (1:1000, Abcam, UK), Bax (1:1000, Abcam, UK), mTOR (1:2000, Abcam, UK), ULK1 (1:1000, Abcam, UK), MMP-1 (1:1000, Abcam, UK), MMP-13 (1:2000, Abcam, UK), ADAMTS-4 (1:200, Abcam, UK), ADAMTS-5 (1:250, Abcam, UK) and GAPDH (1:2500, Abcam, UK) antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies were incubated for an hours at room temperature. Immunoreactive bands were visualized by chemiluminescence (Pierce ECL). The resulting auto-radiograms were then analyzed by densitometry. Equal loading of proteins was qualified by detecting GAPDH levels. Quantification was performed with Image J software.

RNA transfection

The chondrocytes grown to 40% confluence in a six-well plate were transfected with FOXO3 siRNA (Cell signalling technology, Danvers, MA, USA) using the Lipofectamine 2000 transfection reagent (Invitrogen) as our previous described[21]. All the experiments were carried out 48 h after transfection. Knockdown of FOXO3 was confirmed by western blot analysis. The transfected cells were then exposed to serum-deprivation medium for 24 h in the presence or absence of Dex (10^{-6} M).

Flow cytometry

After being transfected with SiFOXO3 RNA, the chondrocytes were treated with 10^{-6} M Dex in serum-free medium for 24 h. Then the apoptosis incidence of the chondrocytes was detected by an Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). The samples were then tested by a fluorescence-activated cell sorter (Beckman Coulter, Miami, FL, USA). According to the user manual, early apoptosis chondrocytes were defined as Annexin V positive and PI negative cells, and late apoptosis chondrocytes were defined as both Annexin V and PI positive cells.

Real-time PCR

The RNA of chondrocytes was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's instructions, 400 ng of total RNA was reverse-transcribed into complementary DNA using the PrimeScript RT reagent kit (Takara RR036A, Shiga, Japan). The expression of MMPs and aggrecanase genes was tested by real-time PCR using SYBR Premix Ex Taq (Takara, Shiga, Japan) and an ABI Prism 7500 sequence detection system (Applied Bio-systems, Foster City, CA, USA). The thermal

Table I
The phenotypes of FAI chondrocytes

	Normal chondrocytes	Chondrocytes from FAI patients	*P* value
Collagen type I	2.95 ± 0.52	3.01 ± 0.56	0.976
Collagen type II	2.51 ± 0.36	2.48 ± 0.48	0.802

The gene relative expressions were normalized to GAPDH. Data are reported as scatter plot and mean values obtained from each experiment. The real-time PCR results showed that there was no difference in the mRNA expression of collagen type I and II between the normal chondrocytes and FAI chondrocytes using independent *t*-test.

Table II
Primer of real-time PCR

Gene	Primer	Sequence (5′ to 3′)
MMP1	MMP1 F	ACAACTGCCAAATGGGCTTGA
	MMP1 R	CTGTCCCTGAACAGCCCAGTACTTA
MMP13	MMP13 F	CTGGGCCAGGGATTAATGGAG
	MMP13 R	CAATTTCATGAGCAGCAACGAGA
ADAMTS4	ADAMTS4 F	ACTATCCCGGCTGCCCTTT
	ADAMTS4 R	TGAGTCTTAGCATGAGGCAGCAA
ADAMTS5	ADAMTS5 F	AATGCACTTCAGCCACCATCA
	ADAMTS5 R	TCGTAGGTCTGTCCTGGGAGTTC
Collagen type I	COL1A1 F	ATGCCTGGTGAACGTGGT
	COL1A1 R	AGGAGAGCCATCAGCACCT
Collagen type II	COL2A1 F	CCCAGAGGTGACAAAGGAGA
	COL2A1 R	CACCTTGGTCTCCAGAAGGA
GAPDH	GAPDH F	ACCCAGAAGACTGTGGATGG
	GAPDH R	GAGGCAGGGATGATGTTCTG

cycling was performed as previously described[21]. The primers were listed in Table II.

Statistical methods

The results of western blot analysis were repeatedly measured for three times (conducted with samples from three different donors). The other data were expressed as the mean of multiple repeats ± S.D. of six independent experiments (individual samples from every donor were studied in triplicate). Cells from different donors were not pooled in any experiment. Statistical analyses were performed using the SPSS 20 statistical software program (IBM, Armonk, NY, USA). The normality and homogeneity of variance of data were tested before statistical analysis. Statistically significant differences between the groups were determined by one-way repeated measures analysis of variance (ANOVA) with Tukey's post test or Dunnett's test. The nonparametric test (Karuskal Wallis) was used when the conditions for data properties were not fulfilled. The difference in the mRNA expressions between FAI and normal chondrocytes was measured by independent *t*-test. *P*-values<0.05 were considered significant.

Results

The induction of autophagy was in response to Dex-induced increase of ROS

To investigate the effect of Dex on ROS, the intracellular ROS levels were recorded after the treatment of different concentrations Dex (10^{-6} M and 10^{-5} M) in serum deprivation medium. The results showed that Dex could significantly up-regulate the endogenous ROS level [Fig. 1(A)]. According to the guide of autophagy monitoring, the accurate measurement of autophagy activity is to detect the autophagic flux, which can be measured by the difference of LC3-II protein levels in the absence and presence of lysosomal inhibitors[26]. To access the autophagic activity in the current study, we investigated the autophagy flux by detecting the accumulation of LC3II (defined as LC3 net flux) using chloroquine to inhibit lysosomes-mediated proteolysis. To determine the effect of Dex on autophagy flux, the chondrocytes were treated with of Dex (10^{-6} M) in serum free medium in the presence and absence of 50 μM chloroquine (CQ, Sigma). To further detect the role of ROS on Dex-induced autophagy, the chondrocytes were pre-treated with catalase (sigma, 500 U/ml) for 3 h before the treatment of Dex at 37°C. The results of western blot analysis showed that the treatment of Dex increased the autophagy flux [Fig. 1(B) and (C)]. Furthermore, using catalase, the ROS inhibitor, decreased Dex-induced autophagy flux [Fig. 1(B) and (C)]. All the results suggested that Dex induced autophagy in human chondrocytes via a ROS-dependent manner. Besides, Dex increased the expressions of

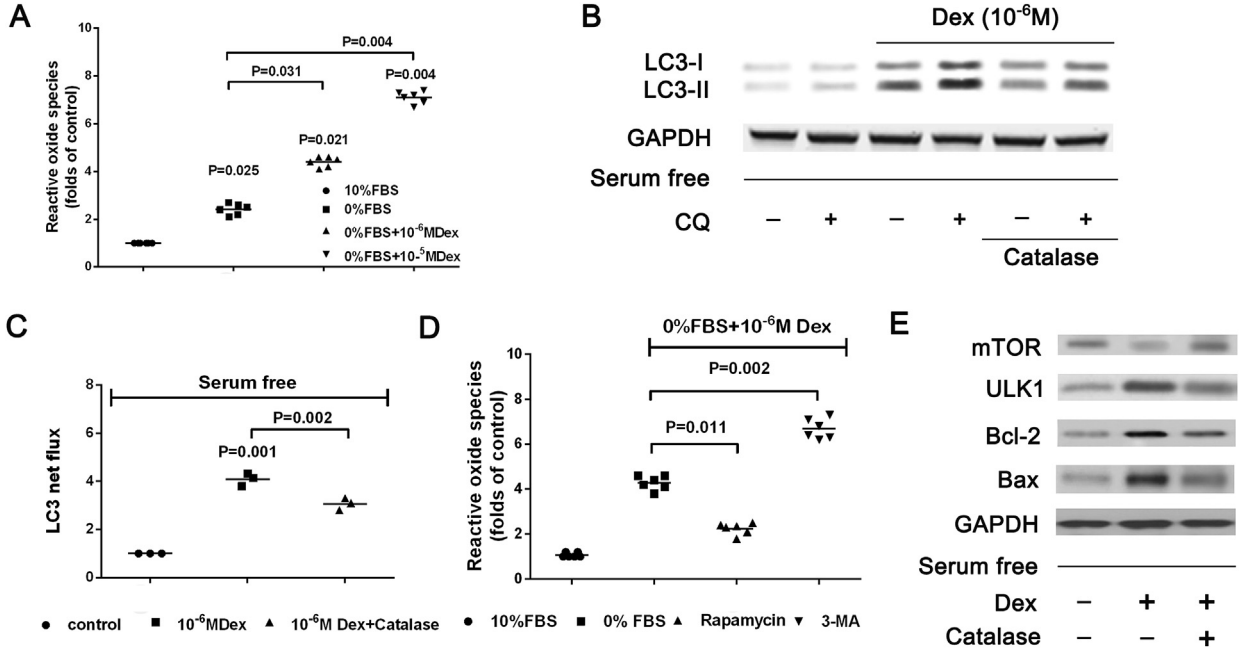

Fig. 1. Dex induced autophagy via increasing intracellular ROS. The chondrocytes were treated with Dex (10^{-5} or 10^{-6} M) in serum deprivation medium in the absence or the presence of catalase for 24 h. (A) Dex increased intracellular ROS level in human chondrocytes ($n = 6$). (B) and (C) Dex (10^{-6} M) increased autophagy flux ($n = 3$). This effect of Dex was inhibited by ROS scavenger, catalase. Autophagy flux was expressed as the subtraction of the amount of LC3-II (LC3 net flux) in the absence of chloroquine (CQ), the autophagy inhibitor from the amount of LC3-II in the presence of CQ for each of the conditions. (D) Rapamycin, the autophagy inducer decreased intracellular ROS level. In contrast, 3-MA, the autophagy inhibitor, increased intracellular ROS level ($n = 6$). (E) Dex up-regulated the expressions of Bax, Bcl-2 as well as ULK1. These effects of Dex were suppressed by catalase, the ROS scavenger. Data are reported as scatter plots and mean values obtained from each experiment.

Bcl-2, ULK1 and Bax in the chondrocytes cultured in serum free medium. These effects of Dex were also suppressed by catalase [Fig. 1(E)]. To further investigate the interaction between ROS and autophagy, we measured the ROS level after the treatments of autophagy inducer and inhibitor. The chondrocytes were pre-treated with rapamycin (10 μM, Sigma–Aldrich, St Louis, MO, USA) or 3-MA (10 nM, Sigma–Aldrich, St Louis, MO, USA) for 2 h before the treatment of Dex. Rapamycin, the autophagy inducer, significantly decreased the intracellular level. In contrast, the ROS level significantly increased after the treatment of Dex when autophagy was suppressed by 3-MA [Fig. 1(D)]. Taken together, the results suggested that autophagy could protect the chondrocytes from increasing levels of ROS. Dex induced autophagy via increasing intracellular ROS level.

Dex up-regulated catabolism of extra-cellular matrix in human chondrocytes

To determine whether Dex could cause degenerative effects in cartilage, we detected the mRNA expression of aggrecanases after the treatment of Dex (10^{-6} M) for 24 h. To detect the role of ROS in Dex-induced changes of matrix metabolism, the chondrocytes were pre-treated with catalase (sigma, 500 U/ml) for 3 h before the treatment of Dex at 37°C. Both the mRNA and proteins expressions of aggrecanases were significantly up-regulated after the treatment of Dex, suggesting that Dex could accelerate the catabolic process of cartilage. Furthermore, this effect of Dex could be repressed by catalase, indicating that Dex increased the expressions of aggrecanases via a ROS signal pathway (Fig. 2).

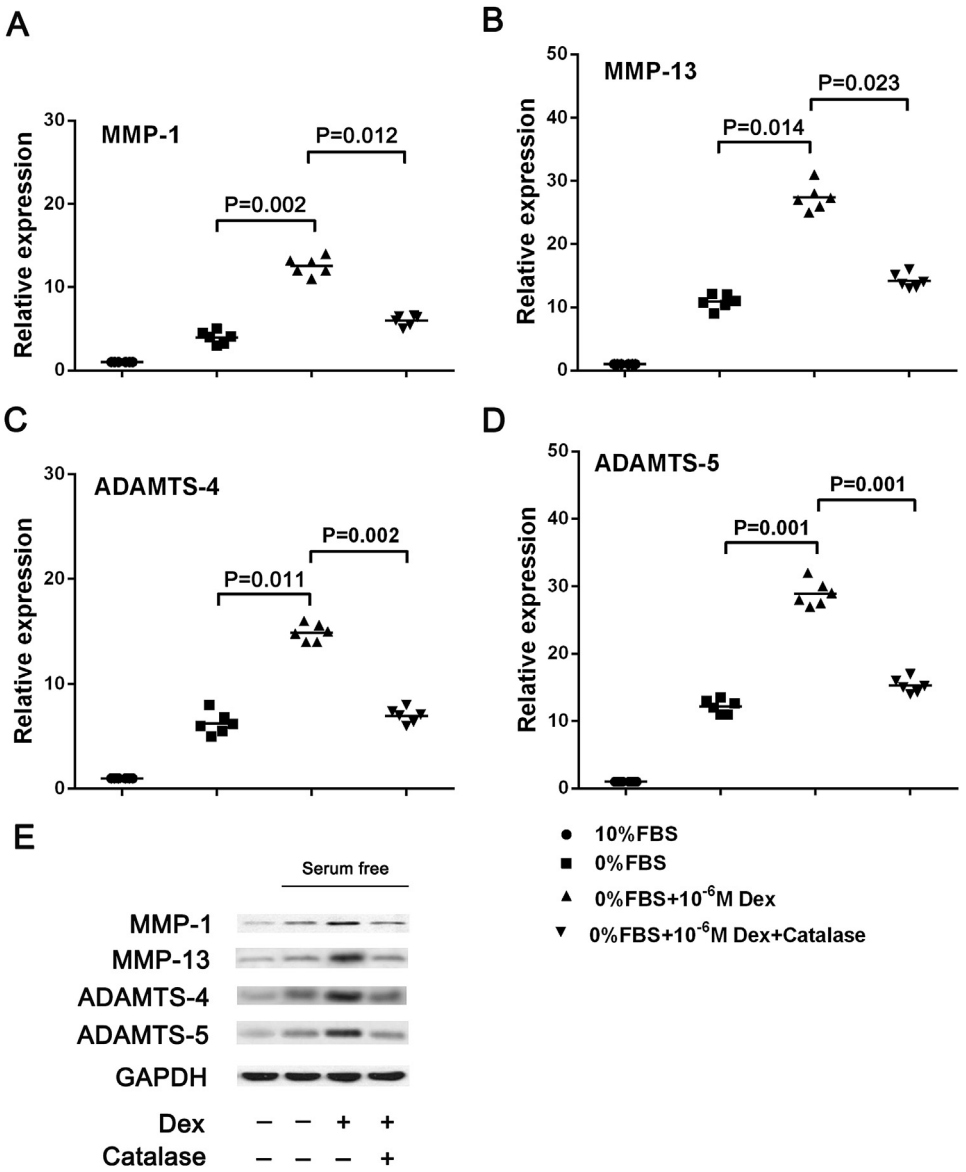

Fig. 2. Dex increased the expressions of aggrecanases. The chondrocytes were cultured in the presence and absence of catalase in serum deprivation medium for 24 h. The chondrocytes cultured in 10%FBS were used as control. (A–D) The results of real-time PCR after the treatment of Dex and catalase ($n = 6$). The data showed that Dex increased the mRNA expressions of MMP-1, MMP-13, ADAMTS-4 and ADAMTS-5. This side-effect of Dex was rescued by catalase. (E) Dex increased the expressions of MMPs and ADAMTSs using western blotting analysis. This effect of Dex was also blocked by catalase. Data are reported as scatter plots and mean values obtained from each experiment.

Dex up-regulated FOXO3 expression through ROS/Akt signal pathway

To investigate the role of ROS/Akt signal in Dex-induced expression of FOXO3, we measured the expression of *p*-Akt as well as *p*-p70S6K after the treatment of Dex. The chondrocytes were pre-treated with catalase (sigma, 500 U/ml) for 3 h before the treatment of Dex at 37°C. Dex treatment significantly increased the expression of *p*-Akt. Furthermore, this effect of Dex on the expression of *p*-Akt was suppressed by catalase, suggested that Dex increased *p*-Akt expression via up-regulation of intracellular ROS level [Fig. 3(A) and (B)]. To further determine the role of Dex-Akt signal pathway in the regulation of FOXO3, we investigated the expression of FOXO3 in the treatment of PI3K/Akt inhibitors, MK-2206 (Selleck chemicals, USA) and LY294002 (Sigma, USA). The chondrocytes were pre-treated with MK-2206 (1 μM) or LY294002 (10 μM) for 2 h before the treatment of Dex. The treatment of Dex augmented the expression of *p*-Akt, Bcl-2, Bax as well as FOXO3 [Fig. 3(C) to (E)]. However, MK-2206 and LY294002, the PI3K/Akt inhibitors, blocked the expression of FOXO3 in the treatment of Dex, indicating that Dex increased FOXO3 expression through an Akt dependent manner [Fig. 3(C) to (E)]. Taken together, all above results suggested that Dex increased FOXO3 expression via ROS/Akt signal pathway.

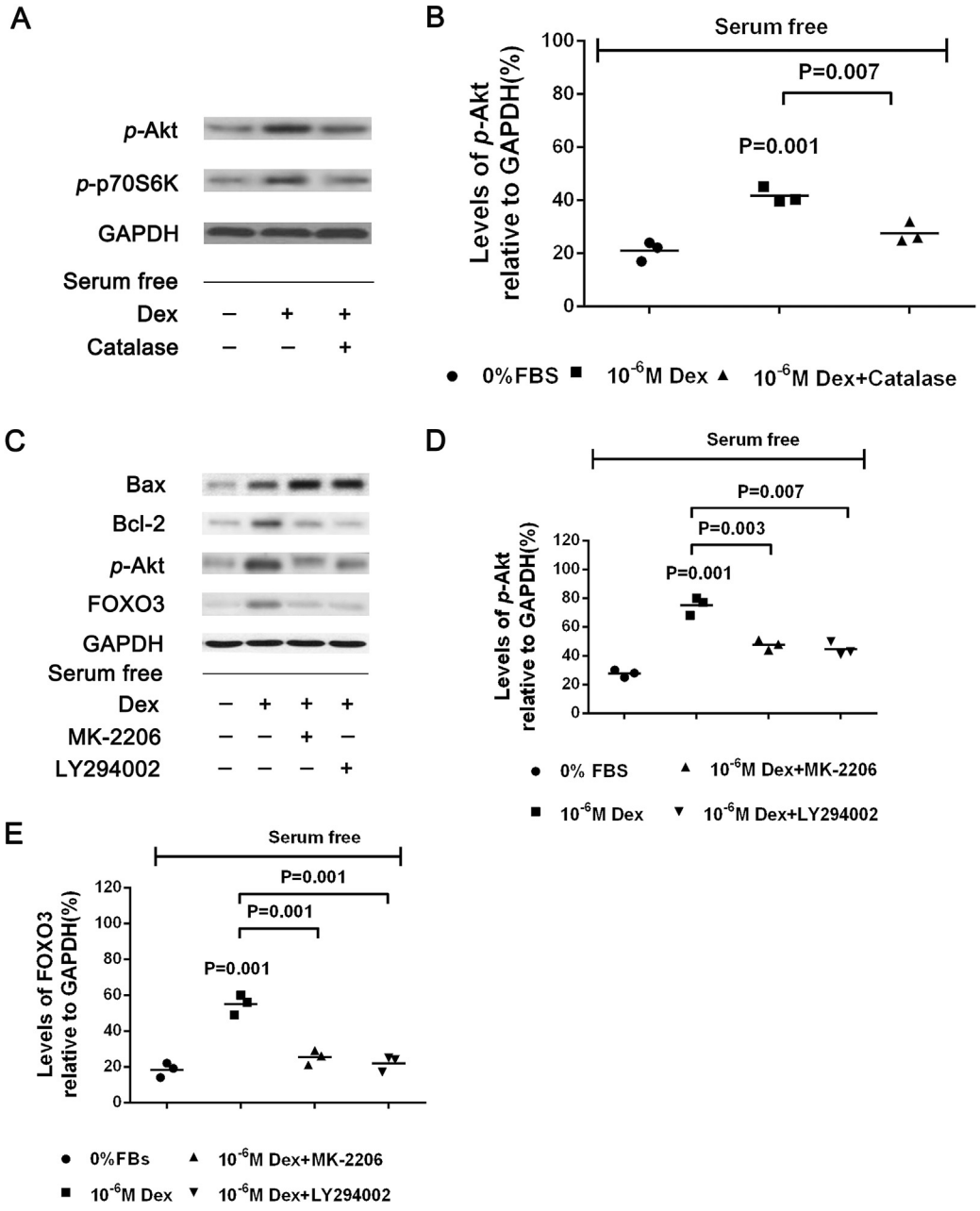

Fig. 3. Dex increased FOXO3 expression via ROS/Akt signal pathway The chondrocytes were cultured in the presence of ROS scavenger or PI3K/Akt inhibitors in serum deprivation medium for 24 h (A) and (B) Dex up-regulated the activity of Akt signal pathway ($n = 3$). This effect of Dex was suppressed by catalase. (C) to (E) MK-2206 and LY294002, the PI3K/Akt inhibitors, repressed Dex-induced up-regulation of FOXO3 and the expression of Bcl-2 ($n = 3$). Suppression of PI3K/Akt signal augmented Dex-induced expression of Bax. Data are reported as scatter plots and mean values obtained from each experiment.

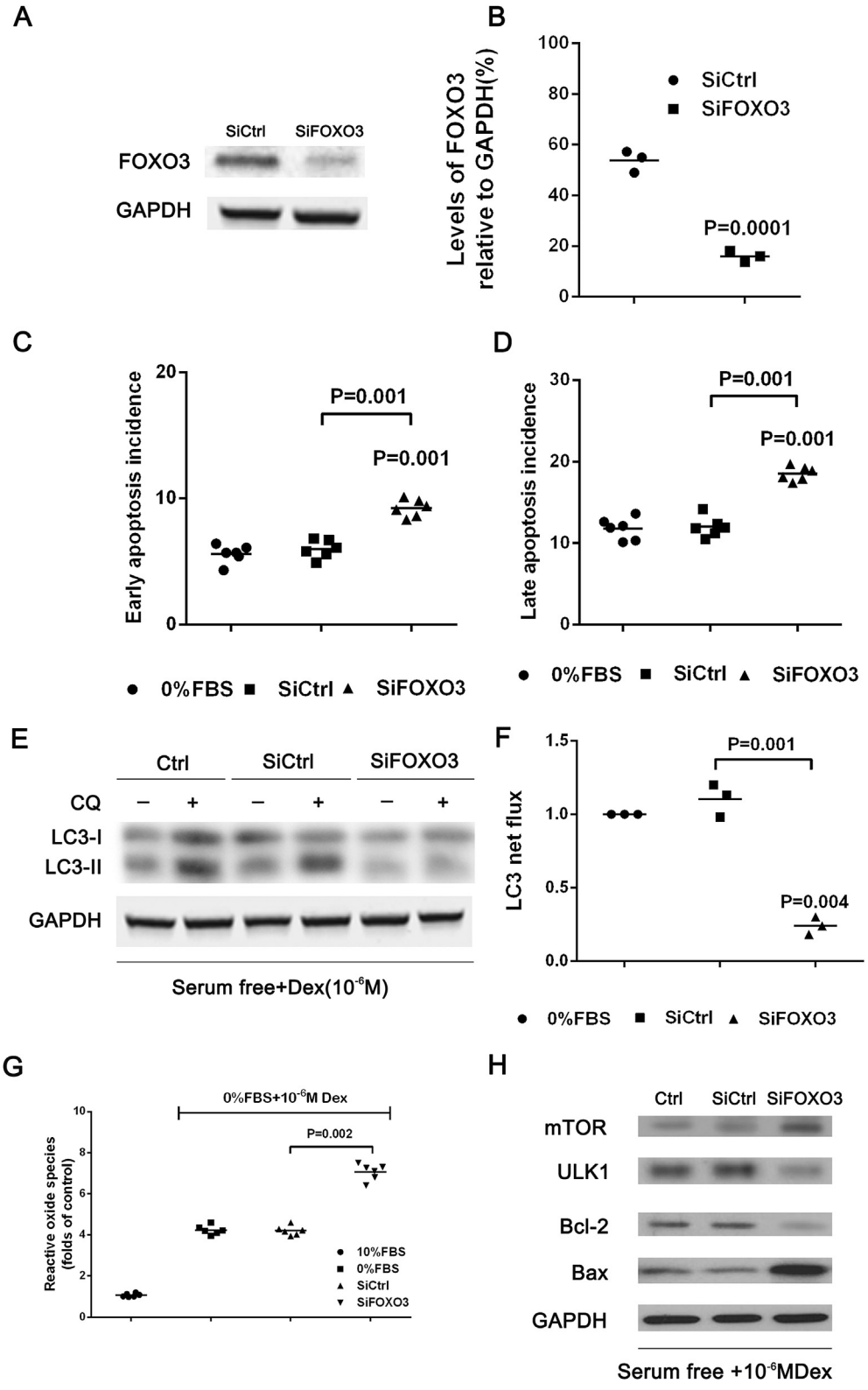

Fig. 4. Dex induced autophagy via FOXO3 expression The chondrocytes were treated with 10^{-6} M Dex in serum deprivation medium for 24 h. (A) and (B) The chondrocytes were transfected with FOXO3 siRNA (+) or control siRNA (−). Knockdown of FOXO3 expression was confirmed by western blot analysis ($n = 3$). (C) and (D) Silencing of FOXO3 resulted in an increase of both early and late apoptosis incidences in human chondrocytes after the treatment of Dex ($n = 6$). (E) and (F) Knockdown of FOXO3 associated with the suppression of autophagy flux, which was expressed as the subtraction of the amount of LC3-II (LC3 net flux)in the absence of CQ from the amount of LC3-II in the presence of CQ for each of the conditions. ($n = 3$). (G) and (H) Silencing FOXO3 increased the intracellular ROS level and the expression of Bax as well as mTOR in the treatment of Dex. The expressions of ULK1 and Bcl-2 were suppressed by FOXO3 silencing. Data are reported as scatter plots and mean values obtained from each experiment.

Dex induced autophagy through up-regulation of FOXO3 expression

To investigate the effect of FOXO3 on Dex-induced autophagy, RNA interference technique was used for silencing the expression of FOXO3 [Fig. 4(A) and (B)]. After the treatment of Dex for 24 h in serum free medium, the western blot analysis results showed that silencing of FOXO3 significantly decreased the LC3 net flux, suggested that Dex increased autophagy activity via up-regulation of FOXO3 [Fig. 4(E) and (F)]. Furthermore, silencing FOXO3 resulted in an up-regulation of both early apoptosis and late apoptosis incidence of chondrocytes, suggested that FOXO3 had a protective effect during the treatment of Dex [Fig. 4(C) and (D)]. Silencing of FOXO3 also increased the intracellular level of ROS and expression of Bax as well as mTOR [Fig. 4(G) and (H)]. The expressions of UKL1 and Bcl-2 were suppressed by FOXO3 silencing. All above results suggested that Dex induced autophagy via ROS/Akt/FOXO3 signal pathway.

Discussion

Dex has been used in the management of OA and RA for decades[2,27,28]. However, there has been a growth body of evidences suggested that Dex might have serious side effects in collagen-producing tissue. Therefore, there have been concerns about the toxic effect of Dex on chondrocytes[27,29,30]. Recently, there was a study demonstrated that autophagy, a catabolic process of energy recycling, could protect chondrocytes from Dex-induced apoptosis[25]. Nevertheless, the molecular mechanisms of Dex-induced autophagy have been not fully elucidated. The work of the current study showed that Dex could increase the intracellular ROS level and consequently induce apoptosis in human chondrocytes. Administration of Dex also resulted in an increase in the expression of MMPs and ADAMTSs via ROS-dependent manner. These findings suggested that Dex could advance the degenerative process of the cartilage via increasing intracellular ROS level. Meanwhile, as a defense mechanism, autophagy was induced in response to Dex-induced increase of ROS. Increased ROS level also up-regulated the expressions of Akt and FOXO3. Silencing FOXO3 resulted in suppression of autophagy. Therefore, our finding suggested that Dex induced autophagy through a ROS/Akt/FOXO3 signal pathway in human chondrocytes (see Fig. 5).

Chondrocytes survived in a hypoxia and avascular environment[31]. Therefore, anaerobic glycolysis is the key mechanism of energy metabolism in chondrocytes. Despite ROS was an important intracellular second messenger, excessive level of ROS might disturb anaerobic metabolism of chondrocytes and disrupt the homeostasis of the cartilage[9,12,14]. Actually, increasing level of ROS has involved in the aging and degenerative process of the cartilages. Several studies reported that ROS could increase the apoptosis incidence and extracellular matrix degradation in chondrocytes[8,15,32]. Accumulation of intracellular ROS could damage the mitochondria and cause oxidative stress. Recently, there were studies reported that GC could dysregulate mitochondria and increase oxidative stress in osteoblasts and chondrocytes[6,8]. Based on these results, we tested that whether Dex had a toxic effect on chondrocytes via increasing the ROS level. In the current study, our results showed that GC could increase ROS and induce cell death in human chondrocytes. Moreover, administration of Dex increased the expressions of aggrecanases and apoptosis incidence in human chondrocytes. These effects of Dex could be repressed by catalase, a common ROS scavenger. Therefore, our results suggested that Dex could accelerate the degenerative effect through increasing the intracellular ROS level.

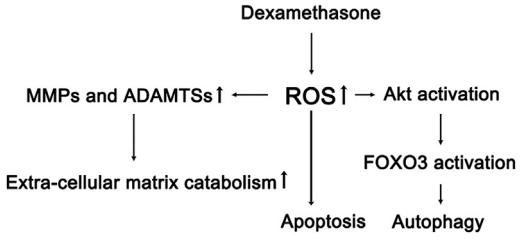

Fig. 5. Dex induced autophagy via ROS/Akt/FOXO3 signal pathway. Dex increased the intracellular level of ROS. ROS, in turns, activated Akt and FOXO3 and resulted in increasing the autophagic flux to protect the chondrocytes from Dex-induced increase of ROS. Suppression of FOXO3 resulted in inhibition of autophagic activity and therefore increased the intracellular ROS level. Dex-induced up-regulation of ROS also augmented the catabolic metabolism of extra-cellular matrix.

Autophagy had involved in the pathological process of OA responsible to ROS/RNS (Reactive nitric species)[21,23]. Previously, our results demonstrated that nitric oxide (NO), a common RNS, could inhibit autophagy and decrease the cell viability in human meniscal cells[21]. There has been evidence showed that ROS also interacted with autophagy during the degenerative process of cartilage[23,33]. Wu *et al.*[33] found that OA chondrocytes had lower autophagic level and higher level of ROS production compared with the normal chondrocytes. Sasaki *et al.*[23] found that induction of autophagy could decrease the intracellular ROS level. In accordance with their results, our study showed that autophagy was induced in response to the Dex-induced up-regulation of ROS level in human chondrocytes. Increased level of ROS could cause oxidative stress and induction of cell death in chondrocytes[12]. Several defense mechanisms have been developed to protect cells against oxidative stress, such as autophagy[34]. Induction of autophagy could decrease the intracellular ROS level[35]. In contrast, inhibition of autophagy resulted in an increase of both intracellular ROS and apoptosis rate after the treatment of Dex. Taken together, our results showed that the induction of autophagy protected chondrocytes from Dex-induced apoptosis via decreasing ROS level.

As a common downstream gene of Akt, FOXO transcription factors have involved in cell proliferation and survival in a variety of tissues[36,37]. In human, FOXOs had bdegenerative processesciate with aging and degenerative process of cartilage[16–18,38]. Akasaki *et al.*[16] reported the reduced expression of FOXO1 and FOXO3 in human OA chondrocytes. Their results indicated that decreased expression of FOXOs would increase the intracellular ROS level and ROS-induced apoptosis[17]. In the current study, our data showed that Dex increased intracellular ROS level, autophagy markers as well as FOXO3 expression. Silencing FOXO3 resulted in reduction of autophagy and cell viability of chondrocytes after the treatment of Dex. Furthermore, silencing FOXO3 also increased the intracellular ROS level. Actually, FOXO3 tightly associated with the regulation of ROS scavenger genes, such as GPX-1 and SOD2[36]. Therefore, knockdown of FOXO3 also resulted in an increase of ROS level. Taken together, our results showed that FOXO3 antagonist increased ROS level by inducing autophagy.

In conclusion, our resulted showed Dex could up-regulate the intracellular ROS level and degradation of matrix in human chondrocytes. These findings suggested that long-term administration of Dex could cause degenerative changes in cartilage. Induction of autophagy was a defense mechanism of chondrocytes in response to Dex-induced increase of ROS. Furthermore, our data showed that the silence of FOXO3 decreased autophagic activity in chondrocytes, indicating that FOXO3 decreased ROS level through up-regulation of autophagy. In summary, these findings suggested that Dex induce autophagy via ROS-Akt-FOXO3 signal pathway.

Author contributions

CS, GQC and JPP conceived of the study, and participated in its design and coordination. CS, GQC and JPP carried out all the experiments and performed quantitative analysis. All authors read and approved the final manuscript. XDC had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics approval

This study was conducted with the approval of the review board of Human Subjects Committee at Xin-hua hospital.

Role of funding source

This study was supported by the National Natural Science Foundation of China (81101379, 81472118, 81171705) and the Natural Science Fund of the Shanghai Jiao Tong University School of Medicine (11XJ21022).

Conflict of interest

None.

Reference

1. Cyriax J, Troisier O. Hydrocortone and soft-tissue lesions. Br Med J 1953;2:966–8.
2. Gaujoux-Viala C, Gossec L. When and for how long should glucocorticoids be used in rheumatoid arthritis? International guidelines and recommendations. Ann N Y Acad Sci 2014;1318:32–40.
3. Baschant U, Culemann S, Tuckermann J. Molecular determinants of glucocorticoid actions in inflammatory joint diseases. Mol Cell Endocrinol 2013;380:108–18.
4. Poulsen RC, Carr AJ, Hulley PA. Protection against glucocorticoid-induced damage in human tenocytes by modulation of ERK, Akt, and forkhead signaling. Endocrinology 2011;152:503–14.
5. Ong SL, Whitworth JA. How do glucocorticoids cause hypertension: role of nitric oxide deficiency, oxidative stress, and eicosanoids. Endocrinol Metab Clin North Am 2011;40:393–407. ix.
6. Almeida M, Han L, Ambrogini E, Weinstein RS, Manolagas SC. Glucocorticoids and tumor necrosis factor alpha increase oxidative stress and suppress Wnt protein signaling in osteoblasts. J Biol Chem 2011;286:44326–35.
7. Baschant U, Lane NE, Tuckermann J. The multiple facets of glucocorticoid action in rheumatoid arthritis. Nat Rev Rheumatol 2012;8:645–55.
8. Grishko V, Xu M, Ho R, Mates A, Watson S, Kim JT, *et al*. Effects of hyaluronic acid on mitochondrial function and mitochondria-driven apoptosis following oxidative stress in human chondrocytes. J Biol Chem 2009;284:9132–9.
9. Milner PI, Wilkins RJ, Gibson JS. The role of mitochondrial reactive oxygen species in pH regulation in articular chondrocytes. Osteoarthritis Cartilage 2007;15:735–42.
10. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell 2012;48:158–67.
11. Sarsour EH, Kumar MG, Chaudhuri L, Kalen AL, Goswami PC. Redox control of the cell cycle in health and disease. Antioxid Redox Signal 2009;11:2985–3011.
12. Henrotin Y, Kurz B, Aigner T. Oxygen and reactive oxygen species in cartilage degradation: friends or foes? Osteoarthritis Cartilage 2005;13:643–54.
13. Kim J, Xu M, Xo R, Mates A, Wilson GL, Pearsall AW, *et al*. Mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human OA chondrocytes. Osteoarthritis Cartilage 2010;18:424–32.
14. Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. Curr Drug Targets 2007;8:333–45.
15. Reed KN, Wilson G, Pearsall A, Grishko VI. The role of mitochondrial reactive oxygen species in cartilage matrix destruction. Mol Cell Biochem 2014;397:195–201.
16. Akasaki Y, Hasegawa A, Saito M, Asahara H, Iwamoto Y, Lotz MK. Dysregulated FOXO transcription factors in articular cartilage in aging and osteoarthritis. Osteoarthritis Cartilage 2014;22:162–70.
17. Akasaki Y, Alvarez-Garcia O, Saito M, Carames B, Iwamoto Y, Lotz MK. FoxO transcription factors support oxidative stress resistance in human chondrocytes. Arthritis Rheumatol 2014;66:3349–58.
18. de Isla N, Charif N, Stoltz JF. Are FoxO transcription factors implicated in osteoarthritis? Influence of Diacerhein. Biomed Mater Eng 2010;20:227–33.
19. Kobayashi Y, Furukawa-Hibi Y, Chen C, Horio Y, Isobe K, Ikeda K, *et al*. SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med 2005;16:237–43.
20. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell 2011;147:728–41.
21. Shen C, Yan J, Erkocak OF, Zheng XF, Chen XD. Nitric oxide inhibits autophagy via suppression of JNK in meniscal cells. Rheumatology (Oxford) 2014;53:1022–33.
22. Shen C, Chen XD, Yan J. Nitric oxide inhibits autophagy in human chondrocytes: comment on the article by Sasaki et al. Arthritis Rheum 2013;65:287–8.
23. Sasaki H, Takayama K, Matsushita T, Ishida K, Kubo S, Matsumoto T, *et al*. Autophagy modulates osteoarthritis-related gene expression in human chondrocytes. Arthritis Rheum 2012;64:1920–8.
24. Shen C, Yan J, Jiang LS, Dai LY. Autophagy in rat annulus fibrosus cells: evidence and possible implications. Arthritis Res Ther 2011;13:R132.
25. Liu N, Wang W, Zhao Z, Zhang T, Song Y. Autophagy in human articular chondrocytes is cytoprotective following glucocorticoid stimulation. Mol Med Rep 2014;9:2166–72.
26. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, *et al*. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8:445–544.
27. Conaghan PG. Corticosteroid injections in osteoarthritis: should we care about predicting response? Rheumatology (Oxford) 2013;52:966–7.
28. Charalambous CP. Corticosteroid compared with hyaluronic acid injections for the treatment of osteoarthritis of the knee. J Bone Joint Surg Am 2004;86-A:874. author reply 874.
29. Dragoo JL, Danial CM, Braun HJ, Pouliot MA, Kim HJ. The chondrotoxicity of single-dose corticosteroids. Knee Surg Sports Traumatol Arthrosc 2012;20:1809–14.
30. Braun HJ, Wilcox-Fogel N, Kim HJ, Pouliot MA, Harris AH, Dragoo JL. The effect of local anesthetic and corticosteroid combinations on chondrocyte viability. Knee Surg Sports Traumatol Arthrosc 2012;20:1689–95.
31. Giannoni P, Cancedda R. Articular chondrocyte culturing for cell-based cartilage repair: needs and perspectives. Cells Tissues Organs 2006;184:1–15.
32. Grishko VI, Ho R, Wilson GL, Pearsall AW. Diminished mitochondrial DNA integrity and repair capacity in OA chondrocytes. Osteoarthritis Cartilage 2009;17:107–13.
33. Wu C, Zheng J, Yao X, Shan H, Li Y, Xu P, *et al*. Defective autophagy in chondrocytes with Kashin-Beck disease but

higher than osteoarthritis. Osteoarthritis Cartilage 2014;22:1936–46.
34. Kiffin R, Bandyopadhyay U, Cuervo AM. Oxidative stress and autophagy. Antioxid Redox Signal 2006;8:152–62.
35. Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 2007;17:422–7.
36. Lam EW, Brosens JJ, Gomes AR, Koo CY. Forkhead box proteins: tuning forks for transcriptional harmony. Nat Rev Cancer 2013;13:482–95.
37. Rokutanda S, Fujita T, Kanatani N, Yoshida CA, Komori H, Liu W, *et al.* Akt regulates skeletal development through GSK3, mTOR, and FoxOs. Dev Biol 2009;328:78–93.
38. Carames B, Kiosses WB, Akasaki Y, Brinson DC, Eap W, Koziol J, *et al.* Glucosamine activates autophagy *in vitro* and *in vivo*. Arthritis Rheum 2013;65:1843–52.